Hepatitis C Virus Particle Assembly Involves Phosphorylation of NS5A by the c-Abl Tyrosine Kinase

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Background: HCV NS5A regulates viral RNA replication and virus particle assembly.

Results: Phosphorylation of NS5A by c-Abl is required for efficient production of infectious HCV particles but not for viral RNA replication.

Conclusion: HCV particle assembly involves tyrosine phosphorylation of NS5A.

Significance: This study provides the first evidence for the importance of NS5A tyrosine phosphorylation in the HCV life cycle.

ABSTRACT

Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is thought to regulate the replication of viral RNA and the assembly of virus particles in a serine/threonine phosphorylation-dependent manner. However, the host kinases that phosphorylate NS5A are not fully identified. Here, we show that HCV particle assembly involves phosphorylation of NS5A by the c-Abl tyrosine kinase. Pharmacological inhibition or knockdown of c-Abl reduces the

production of infectious HCV (J6/JFH1) particles in Huh-7.5 cells, without markedly affecting viral RNA translation and replication. NS5A is tyrosine-phosphorylated in HCV-infected cells, and this phosphorylation is also reduced by knockdown of c-Abl. Mutational analysis reveals that NS5A tyrosine phosphorylation is dependent, at least in part, on Tyr^{330} (Tyr^{2306} in polyprotein numbering). Mutation of this residue to phenylalanine reduces the production of infectious HCV particles, but does not affect the replication of the JFH1 subgenomic replicon. These findings suggest that c-Abl promotes HCV particle assembly by phosphorylating NS5A at Tyr^{330}.

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus of the family *Flaviviridae* (1-3). Following entry into the host cell, the RNA genome of HCV is translated into a polyprotein of ~3,000 amino acids. The polyprotein is co- and post-translationally cleaved by viral and host proteases to yield three structural (core, E1, and E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. By definition, the structural proteins are incorporated into virus particles. The nonstructural proteins, on the other hand, form RNA replication complexes on ER-derived membranous compartments (4). Many of the nonstructural proteins also facilitate the assembly of virus particles.

Recent evidence suggests that HCV particle assembly is initiated near the interface between the ER membrane and cytosolic lipid droplets (LDs), organelles for the storage of neutral lipids (5-7). After proteolytic maturation at the ER membrane, the capsid protein core traffics to the surface of LDs (8), where it binds to viral RNA. The resulting nucleocapsids acquire envelopes by budding into the ER lumen and mature into virus particles. The virus particles are released from the host cell through the secretory pathway.

NS5A is a non-enzymatic protein that plays a critical but as yet undefined role in RNA replication and particle assembly (9). NS5A consists of an amino-terminal amphipathic α-helix and three structural domains (domains I-III). The amphipathic α-helix anchors NS5A to the cytosolic side of the ER membrane (10, 11). Domains I and II are required at least for RNA replication (12, 13), whereas domain III is required for particle assembly (14). NS5A binds to viral RNA *in vitro* (15, 16) and has therefore been proposed to transport viral RNA from replication complexes to LD-resident core (6, 7).

NS5A is a phosphoprotein that exists in basally phosphorylated (p56) and hyperphosphorylated (p58) forms (17, 18). Studies using HCV subgenomic replicons have proposed that NS5A hyperphosphoyrlation negatively regulates RNA replication (19-21). Although

multiple serine and threonine phosphorylation sites have been identified in NS5A (21, 22), their roles in particle assembly are unknown, except for a serine residue at the carboxy terminus of domain III. This residue, Ser^{457}, is phosphorylated by casein kinase (CK) II *in vitro* (23) and is required for efficient particle assembly (23, 24). A recent study has shown that CK I is also involved in HCV particle assembly through NS5A hyperphosphorylation (25). In addition to serine and threonine phosphorylation, we have reported that overexpressed NS5A is tyrosine-phosphorylated in response to treatment with pervanadate, an inhibitor of protein tyrosine phosphatases (26). Moreover, we and others have reported that NS5A binds to non-receptor protein tyrosine kinases such as c-Src, Fyn, Syk, and c-Abl *in vitro* (26-28). However, whether NS5A is tyrosine-phosphorylated in HCV-infected cells is yet to be determined.

The c-Abl tyrosine kinase was discovered as the cellular homolog of v-Abl, the oncoprotein of the Abelson murine leukemia virus (29), and has since been implicated in human cancers (30). In patients with chronic myeloid leukemia (CML), the gene for c-Abl has been translocated from chromosome 9 to chromosome 22 (31). The derivative chromosome 22, known as the Philadelphia chromosome, directs the expression of the fusion protein Bcr-Abl, the cause of CML. The Abl family kinase inhibitor imatinib has proved effective and well tolerated in the treatment of CML and is considered a paradigm of targeted therapeutics.

The Abl family of non-receptor tyrosine kinases consists of c-Abl and Arg (30). Viruses such as coxsackie B virus (32), vaccinia virus (33), and Ebola virus (34) exploit c-Abl and Arg for their entry or egress (35). Although imatinib has been identified as a potential anti-HCV agent in an unbiased compound screen (36), the role of Abl family kinases in the HCV life cycle is unknown.

In this study, we show that c-Abl is involved in HCV particle assembly. We also show that c-Abl phosphorylates NS5A at Tyr^{330} both *in vitro* and in the context of HCV infection and that this residue is required for efficient particle assembly. Our results suggest that c-Abl promotes HCV particle assembly by phosphorylating NS5A at Tyr^{330}.

EXPERIMENTAL PROCEDURES

Cell Culture—Huh-7.5 human hepatoma cells were kindly provided by Charles M. Rice (The Rockefeller University) and were cultured in DMEM (Wako) supplemented with 10% FBS (Sigma-Aldrich) and 0.1 mM nonessential amino acids (Wako). COS-7 cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS. Transfection was performed using FuGENE6 (Promega).

Antibodies and Reagents—Anti-core

(C7-50) antibody was purchased from Abcam. Anti-c-Abl (8E9) antibody was purchased from BD Pharmingen. Anti-Arg, anti-Myc (9E10), and anti-c-Abl antibodies were purchased from Santa Cruz Biotechnology. Anti-GAPDH (6C5) and anti-phosphotyrosine (4G10) antibodies were purchased from Merck Millipore. Anti-GST (5A7) antibody was purchased from Wako. Anti-Flag antibody was purchased from MBL. Anti-Flag (M2) antibody was purchased from Sigma-Aldrich. Anti-NS5A (9E10) antibody was kindly provided by Charles M. Rice (37). Imatinib mesylate was purchased from Wako.

Plasmids—To produce retroviruses encoding shRNAs against human c-Abl and Arg, the c-Abl target sequence, 5′-GAGTTCTTGAAGCATTTCAAA-3′, and Arg target sequence, 5′-GCTGGAATGTGCTGACCTACT-3′, were cloned into pSuper retro-puro and pSuper retro-hygro (Oligoengine), respectively (38). The sequences of human c-Abl and Arg were amplified by PCR from Huh-7.5 cell cDNAs and cloned into pcDNA3. pcDNA3-murine c-Abl and pcDNA3-murine c-Abl KD (K290M) were kindly provided by Tomoyuki Shishido (Nara Institute of Science and Technology). The sequence of murine c-Abl was amplified by PCR from pcDNA3-murine c-Abl and cloned into pcDNA3-Flag. pEF1A-NS5A (Con1)-Myc-His was described previously (28). Site-directed mutagenesis was used to replace conserved tyrosine residues in NS5A with phenylalanine. pFL-J6/JFH1 was kindly provided by Charles M. Rice (37). Site-directed mutagenesis was used to replace NS5A Tyr^{330} with phenylalanine. EcoRI/Bsp1407I and ClaI/Bsp1407I fragments were cloned into pFL-J6/JFH1 and pSGR-JFH1 (39), respectively. pSGR-JFH1 was kindly provided by Takaji Wakita (National Institute of Infectious Diseases). The sequence of NS5A domain II (amino acids 250-338) was amplified by PCR from pFL-J6/JFH1 and cloned into pGEX-4T-3.

HCV Infection—HCVcc (J6/JFH1) with adaptive mutations (P-47) was described previously (40). Huh-7.5 cells were infected with HCVcc at a multiplicity of infection of 3. Media were replaced with fresh media 4-6 h after infection.

Focus-Forming Assay—To evaluate extracellular infectivity, culture supernatants were collected. To evaluate intracellular infectivity, cells were trypsinized and suspended in culture media. Cells were centrifuged at 2,000 × *g* for 2 min, suspended in culture media, and subjected to three cycles of freezing and thawing. Samples were centrifuged at 2,000 × *g* for 2 min. Supernatants were used to infect naive Huh-7.5 cells. Cells were incubated for 72 h, fixed with methanol for 20 min at –20°C, and stained with anti-core antibody. Foci of infected cells were

manually counted with a fluorescence microscope (IX70, Olympus).

Real-Time PCR—Cells were washed three times with PBS. Total RNA was extracted using a High Pure RNA isolation kit (Roche). One hundred ng of total RNA was subjected to reverse transcription. RNA in culture supernatants was extracted using a QIAamp Viral RNA Mini Kit (Qiagen). Real-time PCR was performed using a SYBR FAST qPCR kit (KAPA Biosystems) and a StepOne Plus real-time PCR system (Life Technologies). Reactions contained HCV-specific forward and reverse primers (5′-CTTCACGCAGAAAGCGTCTA-3′ and 5′-CAAGCACCCTATCAGGCAGT-3′, respectively). The standard curve was constructed using HCV RNA transcribed *in vitro*. To quantify c-Abl and Arg mRNA levels, real-time PCR was performed with c-Abl-specific forward and reverse primers (5′-GGCTGGGTCCCAAGCAA-3′ and 5′-ACACAGGCCCATGGTACCA-3′, respectively) or Arg-specific forward and reverse primers (5′-CTGCAGATGGCAAGGTGTATGT-3′ and 5′-TGTTGTCACCAGCCCATCAG-3′, respectively). The standard curves were constructed using c-Abl and Arg RNA transcribed *in vitro*.

Retroviral Infection—Retroviral packaging was performed using HEK293T cells. Retrovirus-containing culture supernatants were filtered through a 0.45-μm-pore-size filter and used to infect Huh-7.5 cells in the presence of 8 μg/ml polybrene for 8 h. After fresh media were added, cells were incubated for 48 h.

Immunoblotting—Cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 2 μg/ml aprotinin) and centrifuged at 15,000 × *g* for 20 min at 4°C. Immunoprecipitation was performed using protein A Sepharose (GE Healthcare). Immunoprecipitates were subjected to SDS-PAGE. Blots were quantified using ImageJ software.

Purification of GST Fusion Proteins—Competent cells (DH5α) were transformed with pGEX-4T-3-NS5A domain II WT or Y330F and cultured in LB broth. The expression of the GST fusion proteins was induced by adding IPTG to a final concentration of 0.5 mM. LB cultures were incubated for 2 h at 37°C and centrifuged at 5,000 × *g* for 15 min at 4°C. After sonication in PBS containing 1 mM PMSF and 2 μg/ml aprotinin, Triton X-100 was added to a final concentration of 1%. Lysates were cleared by centrifugation and incubated with glutathione Sepharose 4B (GE Healthcare) for 30 min at room temperature. GST fusion proteins were eluted with elution buffer (50 mM Tris [pH 8.0], 10 mM reduced glutathione).

In Vitro Kinase Assay—HEK293T cells

were transfected with pcDNA3-c-Abl-Flag, lysed in lysis buffer, and centrifuged at 15,000 × *g* for 20 min. Immunoprecipitation was performed using anti-Flag antibody agarose (MBL). Immunoprecipitates were incubated with GST-NS5A domain II WT or Y330F in kinase buffer (20 mM Tris [pH 7.4], 1 mM DTT, 10 mM $MgCl_2$, 10 mM ATP) for 15 min at room temperature and subjected to SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antibody.

In Vitro Transcription—In vitro transcription of HCV RNA was performed as described elsewhere (41). Briefly, DNA templates were linearized with XbaI and treated with mung bean nuclease. Linearized templates were treated with proteinase K and purified by ethanol-chloroform extraction. RNA was transcribed *in vitro* using a MEGAscript kit (Ambion) and purified using a High Pure RNA isolation kit. RNA stocks (1 μg/μl) were stored at –80°C. *In vitro* transcription of c-Abl and Arg RNA was performed similarly, except that linearized pcDNA3-c-Abl and pcDNA3-Arg were not treated with mung bean nuclease.

Electroporation—Cells were trypsinized, suspended in culture media, and centrifuged at 2,000 × *g* for 2 min. After washing with PBS, cells were suspended at 1×10^7 cells/ml in PBS. A cell suspension (400 μl) was mixed with 5 μg of RNA in a 4-μm gap cuvette and electroporated at 950 μF and 260 V using a Gene Pulser Xcell electroporation system (Bio-Rad). After a 10 min recovery time, cells were suspended in culture media and plated.

Immunofluorescence—Cells were fixed with 4% paraformaldehyde (PFA) for 30 min, permeabilized with 50 μg/ml digitonin for 5 min, and incubated with primary antibodies overnight at 4°C. Alexa Fluor 488- and 555-conjugated anti-mouse IgG (Cell Signaling Technology) and Alexa Fluor 647-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. LDs were stained with 5 μg/ml BODIPY 493/503 (Molecular Probes). Nuclei were stained with Hoechst 33258 (Wako). Cells were observed with a confocal microscope (TCS SPII, Leica) equipped with a 100× oil-immersion objective. Acquired images were analyzed using ImageJ software.

IP-Real-Time PCR—IP-real-time PCR was performed as described elsewhere (24), with minor modifications. Cells were washed with PBS and incubated in hypotonic buffer (10 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF) for 5 min on ice. NP-40 was added to a final concentration of 1%. Lysates were incubated for 10 min on ice and cleared by centrifuging at 4,000 × *g* for 15 min. Glycerol was added to a final concentration of 5%. Lysates were precleared by incubating with protein A Sepharose for 30 min at room temperature and incubated with anti-Flag

or anti-core monoclonal antibody for 1h. Immunoprecipitation was performed using protein A Sepharose. Immunoprecipitates were washed three times with wash buffer (10 mM Tris [pH 7.6], 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT). Immunoprecipitated core was eluted by incubating with elution buffer (50 mM Tris [pH 8.0], 1% SDS, 10 mM EDTA) for 10 min at 65°C. After incubation with 100 μg of proteinase K for 30 min at 37°C, coprecipitated HCV RNA was extracted using TRIzol LS reagent (Life Technologies) and subjected to reverse transcription and real-time PCR.

Statistical Analysis—Data were analyzed by unpaired Student's *t* test.

RESULTS

c-Abl Is Involved in HCV Particle Assembly—Treatment with the Abl inhibitor imatinib prevents spread of HCV infection in cell culture (36), but does not appear to affect virus entry (42). To determine which step in the HCV lifecycle is blocked by imatinib treatment, Huh-7.5 cells were infected with cell culture-grown HCV (HCVcc) of the J6/JFH1 chimeric genome (37, 40) and were incubated in the presence or absence of imatinib. Extracellular and intracellular virus particles were harvested and quantified by focus-forming assay. Imatinib treatment markedly reduced both extracellular and intracellular infectivity (Fig. 1, *A* and *B*), while only marginally reducing intracellular HCV RNA levels (Fig. 1*C*) and core and NS5A expression (Fig. 1*D*). Imatinib treatment did not significantly affect cell viability (Fig. 1*E*), consistent with a previous study (36). These results indicate that imatinib treatment reduces HCV particle assembly with a minimal effect on viral RNA translation and replication.

Imatinib inhibits c-Abl, Arg, and several other tyrosine kinases (31, 43). To examine whether c-Abl or Arg is required for HCV particle assembly, Huh-7.5 cells stably expressing an shRNA against c-Abl or Arg, or both were established and infected with HCVcc. Knockdown of c-Abl and Arg was confirmed by immunoblotting (Fig. 2*A*). Similar to treatment with imatinib, knockdown of c-Abl reduced extracellular and intracellular infectivity (Fig. 2, *B* and *C*), but did not significantly affect intracellular HCV RNA levels (Fig. 2*D*) or core expression (Fig. 2*E*). These results indicate that c-Abl is involved in HCV particle assembly. In contrast, knockdown of Arg had no significant effect on extracellular or intracellular infectivity (Fig. 2, *B* and *C*). However, because Arg mRNA levels were more than 10 times lower than c-Abl mRNA levels in Huh-7.5 cells (Fig. 2*F*), the possibility cannot be excluded that Arg is also involved in HCV particle assembly.

c-Abl Phosphorylates NS5A at Tyr^{330}—We have previously reported that the SH3 domain of c-Abl binds to NS5A *in vitro* and that

overexpressed NS5A is tyrosine-phosphorylated in response to pervanadate treatment (26). We therefore investigated the possibility that c-Abl promotes HCV particle assembly by phosphorylating NS5A. NS5A was immunoprecipitated from lysates of HCVcc-infected Huh-7.5 cells, and its tyrosine phosphorylation was evaluated by immunoblotting. The p58 form of NS5A was preferentially tyrosine-phosphorylated (Fig. 3*A*). This phosphorylation was reduced by knockdown of c-Abl (Fig. 3*A*). In addition, NS5A of the Con1 isolate was tyrosine-phosphorylated in COS-7 cells when coexpressed with wild-type (WT), but not kinase-dead (KD), c-Abl (Fig. 3*B*). These results suggest that c-Abl is involved in tyrosine phosphorylation of NS5A.

To identify a putative phosphorylation site for c-Abl, tyrosine residues conserved between the Con1 (genotype 1b) and JFH1 (genotype 2a) isolates were mutated to phenylalanine in Con1 NS5A. Of the eight mutations, the Y334F mutation significantly reduced NS5A tyrosine phosphorylation in COS-7 cells (Fig. 3, *C* and *D*). This residue is at the carboxy terminus of domain II and corresponds to Tyr^{330} of JFH1 NS5A (Fig. 3*E*). c-Abl phosphorylated NS5A domain II (JFH1) *in vitro* (Fig. 3*F*). This phosphorylation was reduced by the Y330F mutation (Fig. 3*F*). These results suggest that c-Abl phosphorylates NS5A at Tyr^{330}.

Tyr^{330} of NS5A Is Required for Efficient HCV Particle Assembly—To examine whether Tyr^{330} of NS5A is required for HCV particle assembly, HCV genomic RNAs encoding NS5A WT and Y330F (J6/JFH1 WT and Y330F, respectively) were transcribed *in vitro* and electroporated into Huh-7.5 cells. As expected, the p58 form of NS5A WT was tyrosine-phosphorylated (Fig. 4*A*). The Y330F mutation reduced this phosphorylation (Fig. 4*A*) as well as extracellular and intracellular infectivity (Fig. 4, *B* and *C*). Although the Y330F mutation also slightly reduced intracellular HCV (J6/JFH1) RNA levels (Fig. 4*D*) and NS5A expression (Fig. 4, *A* and *E*), the same mutation did not significantly affect the replication of the JFH1 subgenomic replicon (SGR-JFH1) (Fig. 4, *F* and *G*). These results suggest that Tyr^{330} of NS5A is required for efficient HCV particle assembly.

c-Abl Is Dispensable for the Binding of Core to Viral RNA—NS5A has been proposed to transport viral RNA from replication complexes to LD-resident core, thereby promoting nucleocapsid formation (6, 7). We examined whether tyrosine phosphorylation by c-Abl regulates the subcellular localization of NS5A. The association of NS5A with LDs was comparable between control and c-Abl knockdown cells (Fig. 5, *A* and *B*) and was unaffected by the Y330F mutation (Fig. 5, *C* and *D*). We next examined whether c-Abl is required for the binding of core to viral RNA. Core was

immunoprecipitated from lysates of control and c-Abl knockdown cells, and coprecipitated viral RNA was quantified by real-time PCR. Knockdown of c-Abl slightly increased the amount of coprecipitated viral RNA (Fig. 6*A*), while reducing the amount of viral RNA in culture supernatants (Fig. 6*B*). These results suggest that c-Abl is dispensable for the binding of core to viral RNA.

DISCUSSION

NS5A has long been known to exist in basally phosphorylated (p56) and hyperphosphorylated (p58) forms (17, 18). Although the functional difference between these two forms is unclear, recent studies have implicated the p58 form of NS5A in HCV particle assembly (23, 25). Consistent with these studies, our results suggest that c-Abl promotes HCV particle assembly by phosphorylating the p58 form of NS5A. It is difficult to speculate on the exact role for this phosphorylation since the mechanism by which NS5A facilitates particle assembly remains elusive. Because of its RNA-binding activity, NS5A has been proposed to transport viral RNA to LD-resident core prior to nucleocapsid formation (6, 7). However, our results suggest that phosphorylation of NS5A by c-Abl is dispensable for the binding of core to viral RNA. Rather, the amount of core-bound viral RNA was increased in c-Abl knockdown cells (Fig. 6*A*). This result can be interpreted to mean that knockdown of c-Abl impairs a later step in particle assembly (i.e., nucleocapsid formation or envelopment), thereby preventing core-bound viral RNA from being released as virus particles.

Tyr^{330} of NS5A (Tyr^{2306} in polyprotein numbering) is conserved among all genotypes of HCV (13) and conforms to the preferred c-Abl substrate sequence YXXP (44). We identified this tyrosine residue as a putative phosphorylation site for c-Abl by coexpressing NS5A mutants with c-Abl in COS-7 cells and by *in vitro* kinase assay. Although these systems do not fully represent the situation for NS5A in HCV-infected cells, our results using J6/JFH1 also indicate that NS5A tyrosine phosphorylation is dependent, at least in part, on Tyr^{330}. Tyr^{330} of NS5A is therefore likely to be a direct target of c-Abl in HCV-infected cells. The SH2 domains of Crk and Nck are known to recognize phosphorylated YXXP sequences (45), but do not appear to bind to NS5A in HCV-infected cells (data not shown).

Recent mass spectrometric analyses of NS5A identified multiple serine and threonine but not tyrosine phosphorylation sites (21, 22). Given that NS5A was purified from subgenomic replicon-harboring cells in these analyses, it is conceivable that phosphorylation of NS5A at Tyr^{330} may occur only in virus particle-producing cells.

We showed that the Y330F mutation

did not significantly affect SGR-JFH1 replication (Fig. 4, *F* and *G*). In contrast, a previous study showed that the Y330A mutation abolished SGR-JFH1 replication (13). This difference may indicate that although NS5A Tyr^{330} phosphorylation is dispensable for RNA replication, the structure of the carboxy terminus of domain II influences the efficiency of RNA replication.

HCV exploits various host kinases to complete its life cycle (46). For example, HCV RNA replication requires c-Src activity (47). We showed that imatinib treatment reduced intracellular viral RNA levels by ~20% (Fig. 1*C*). This may be because imatinib weakly inhibits c-Src (48) or other kinases involved in HCV RNA replication.

The identification of c-Abl as a host factor for HCV particle assembly may have clinical implications. HCV has infected ~3% of the world's population and is a major cause of liver cirrhosis and hepatocellular carcinoma (49). This has prompted the development of numerous anti-HCV agents that target viral proteins such as the NS3-4A protease and NS5A (50) or host proteins such as cyclophilin A (51). Imatinib and more potent Abl inhibitors are already in clinical use as targeted therapeutic agents for CML (31). Although the spread of HCV infection may not necessarily require particle assembly (52), our results suggest the potential of Abl inhibitors as therapeutic agents for HCV infection.

c-Abl can contribute to liver tumorigenesis when activated by oncogenic receptor tyrosine kinases (53). However, it is unknown whether c-Abl is activated in HCV-induced hepatocellular carcinoma.

Serine/threonine phosphorylation of the NS5A/NS5 proteins has been found not only in HCV but also in other members of the family *Flaviviridae* such as bovine viral diarrhea virus, yellow fever virus (54), and dengue virus (55). In addition, some of them require Src family kinases for the assembly and maturation of virus particles (56, 57). It would be interesting to determine whether tyrosine phosphorylation of the NS5A/NS5 proteins is a conserved feature of the family *Flaviviridae*.

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Author contributions: SY conceived, designed, and performed the experiments, analyzed the data, and wrote the paper. KT performed the experiments and analyzed the data. KC, CH, and HY analyzed the data. XS and HH contributed reagents/materials/analysis tools. KS conceived and designed the experiments, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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The abbreviations used are: HCV, hepatitis C virus; NS5A, nonstructural protein 5A; LD, lipid droplet; CK, casein kinase; CML, chronic myeloid leukemia.

FIGURE 1. **Treatment with imatinib reduces HCV particle assembly.** *A-D*, Huh-7.5 cells were infected with HCVcc (J6/JFH1) and treated with DMSO (vehicle) or imatinib (10 μM) for 72 h. *A*, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. *B*, extracellular and intracellular infectivity (FFU/ml) was quantified by counting core-positive foci and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.01$. *C*, intracellular HCV RNA levels were quantified by real-time PCR and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.05$. *D*, the expression levels of core and NS5A were evaluated by immunoblotting. The intensity ratio of core or NS5A to GAPDH was calculated from blots and normalized to the control value. *E*, Huh-7.5 cells were

treated with DMSO or imatinib (10 μM) for 72 h. Cell viability was assessed by trypan blue assay. Data represent the mean ± SD (n=3). NS, not significant.

FIGURE 2. **Knockdown of c-Abl reduces HCV particle assembly.** *A-E*, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus together with a control or Arg shRNA-encoding retrovirus. *A*, the expression levels of c-Abl and Arg were evaluated by immunoblotting. *B-E*, cells were infected with HCVcc (J6/JFH1) and incubated for 48 h. *B*, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. *C*, extracellular and intracellular infectivity (FFU/ml) was quantified by counting core-positive foci and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.01$. *D*, intracellular HCV RNA levels were quantified by real-time PCR and normalized to control values. Data represent the mean ± SD (n=3). NS, not significant. *E*, the level of core expression was evaluated by immunoblotting. *F*, c-Abl and Arg mRNA levels in Huh-7.5 cells were quantified by real-time PCR and normalized to c-Abl values. Data represent the mean ± SD (n=3). *, $P < 0.01$.

FIGURE 3. **c-Abl phosphorylates NS5A at Tyr^{330}.** *A*, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus. Cells were infected with HCVcc (J6/JFH1) and incubated for 96 h. Cells were lysed and subjected to immunoprecipitation with anti-NS5A antibody. The level of phosphorylated NS5A was evaluated by anti-phosphotyrosine (p-Tyr) immunoblotting. Black arrowheads indicate the positions of NS5A. *B*, COS-7 cells were transfected with a Myc-tagged NS5A (Con1) expression vector together with a c-Abl wild-type (WT) or kinase-dead (KD) expression vector. Cells were lysed and subjected to immunoprecipitation with anti-Myc antibody. The level of phosphorylated NS5A was evaluated by anti-p-Tyr immunoblotting. *C*, COS-7 cells were transfected with a c-Abl expression vector together with a Myc-tagged NS5A expression vector or expression vectors for the indicated mutants. The level of NS5A tyrosine phosphorylation was evaluated as in *B*. *D*, the intensity ratio of p-Tyr to Myc was calculated from blots in *C* for each mutant and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.01$. *E*, domain structure of NS5A. Numbers indicate amino acid positions on the sequence of JFH1 NS5A. Sequences of the carboxy terminus of domain II (the JFH1 and Con1 isolates) are shown below. AH, amphipathic helix. *F*, Flag-tagged c-Abl was transiently expressed in HEK293T cells and immunoprecipitated with anti-Flag antibody. Immunoprecipitates were incubated with GST-tagged NS5A domain II (JFH1) WT or Y330F in kinase buffer. The level of

phosphorylated NS5A was evaluated by anti-p-Tyr immunoblotting.

FIGURE 4. **The NS5A Y330F mutation reduces HCV particle assembly.** *A*-*E*, Huh-7.5 cells were electroporated with HCV genomic RNAs encoding NS5A WT and Y330F (J6/JFH1 WT and Y330F, respectively) and incubated for 48 h. *A*, cells were lysed and subjected to immunoprecipitation with anti-NS5A antibody. The level of phosphorylated NS5A was evaluated by anti-phosphotyrosine (p-Tyr) immunoblotting. Black arrowheads indicate the positions of NS5A. *B*, extracellular and intracellular virus particles were harvested and used to infect naive Huh-7.5 cells. Cells were stained for core (green) and nuclei (blue). Scale bar, 200 μm. *C*, extracellular and intracellular infectivity (FFU/ml) was quantified by counting core-positive foci and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.01$. *D*, intracellular HCV RNA levels were quantified by real-time PCR and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.01$. *E*, the level of NS5A expression was evaluated by immunoblotting. *F* and *G*, Huh-7.5 cells were electroporated with HCV subgenomic replicon RNAs encoding NS5A WT and Y330F (SGR-JFH1 WT and Y330F, respectively) and incubated for 48 h. *F*, intracellular HCV RNA levels were quantified as in *D*. NS, not significant. *G*, the level of NS5A expression was evaluated by immunoblotting.

FIGURE 5. **Knockdown of c-Abl does not affect the association of NS5A with LDs.** *A* and *B*, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus. Cells were infected with HCVcc (J6/JFH1) and incubated for 48 h. *A*, cells were stained for NS5A (red), LDs (green), and nuclei (blue). Boxed regions are shown enlarged in right panels. Scale Bar, 10 μm. *B*, the percentage of LDs associated with NS5A in each cell was calculated. Data represent the mean ± SD. Fifteen cells were analyzed. NS, not significant. *C* and *D*, Huh-7.5 cells were electroporated with HCV genomic RNAs encoding NS5A WT and Y330F (J6/JFH1 WT and Y330F, respectively) and incubated for 48 h. The subcellular localization of NS5A was analyzed as in *A* and *B*.

FIGURE 6. **Knockdown of c-Abl increases the amount of core-bound viral RNA in HCV-infected cells.** *A* and *B*, Huh-7.5 cells were infected with a control or c-Abl shRNA-encoding retrovirus. Cells were infected with HCVcc (J6/JFH1) and incubated for 48 h. *A*, cells were lysed and subjected to immunoprecipitation with anti-core antibody. Coprecipitated viral RNA was quantified by real-time PCR and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.05$. The level of

immunoprecipitated core was evaluated by immunoblotting. *B*, HCV RNA levels in culture supernatants were quantified by real-time PCR and normalized to control values. Data represent the mean ± SD (n=3). *, $P < 0.05$.

FIGURE 1

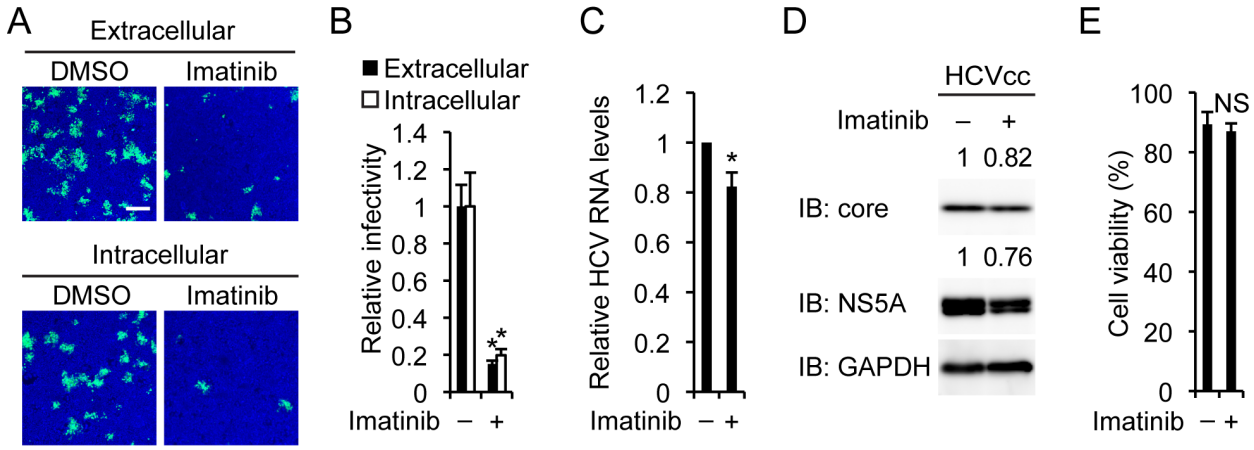

A
Extracellular
DMSO
Imatinib
Intracellular
DMSO
Imatinib
B
Extracellular
Intracellular
Relative infectivity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Imatinib – +
C
Relative HCV RNA levels
1.2
1
0.8
0.6
0.4
0.2
0
*
Imatinib – +
D
HCVcc
Imatinib – +
1 0.82
IB: core
1 0.76
IB: NS5A
IB: GAPDH
E
Cell viability (%)
100
80
60
40
20
0
NS
Imatinib – +

FIGURE 2

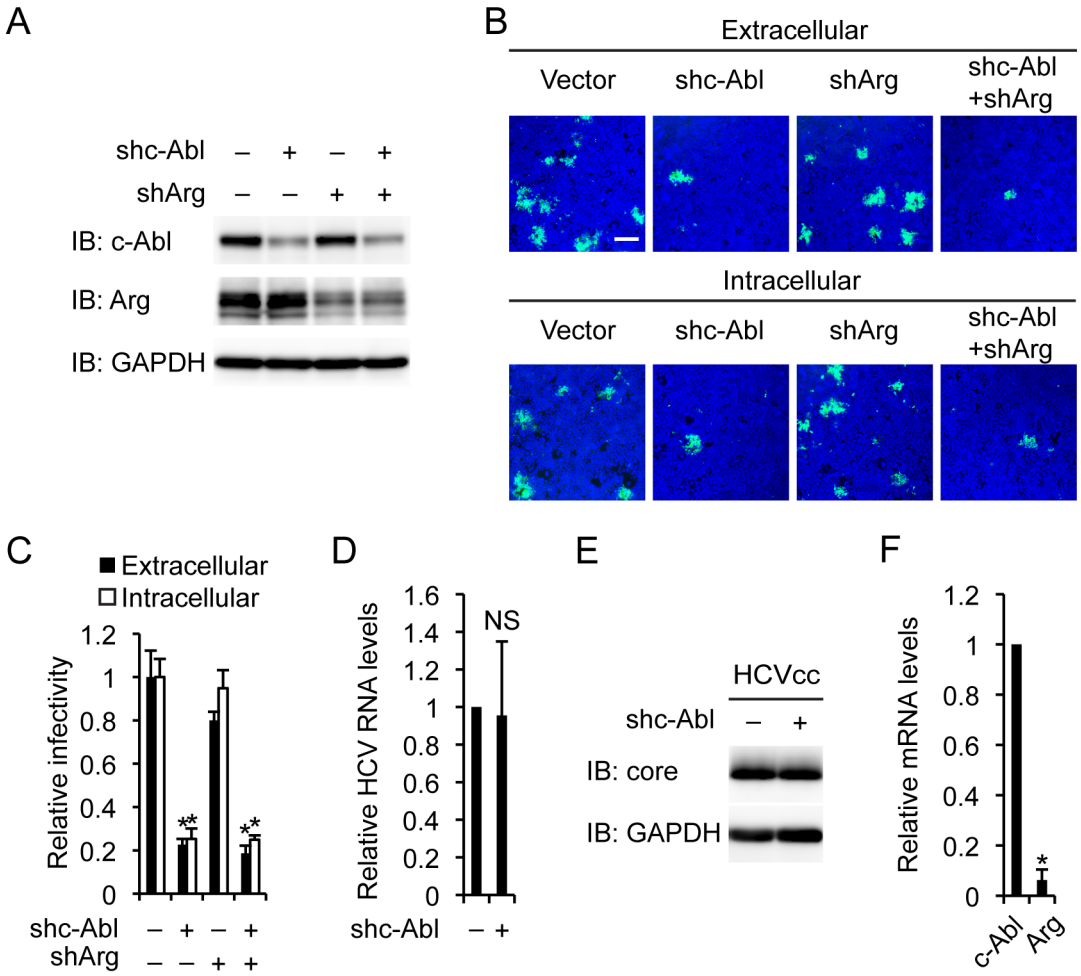

A
shc-Abl – + – +
shArg – – + +
IB: c-Abl
IB: Arg
IB: GAPDH
B
Extracellular
Vector
shc-Abl
shArg
shc-Abl +shArg
Intracellular
Vector
shc-Abl
shArg
shc-Abl +shArg
C
Extracellular
Intracellular
Relative infectivity
1.2
1
0.8
0.6
0.4
0.2
0
**
**
shc-Abl – + – +
shArg – – + +
D
Relative HCV RNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
shc-Abl – +
E
HCVcc
shc-Abl – +
IB: core
IB: GAPDH
F
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
*
c-Abl
Arg

FIGURE 3

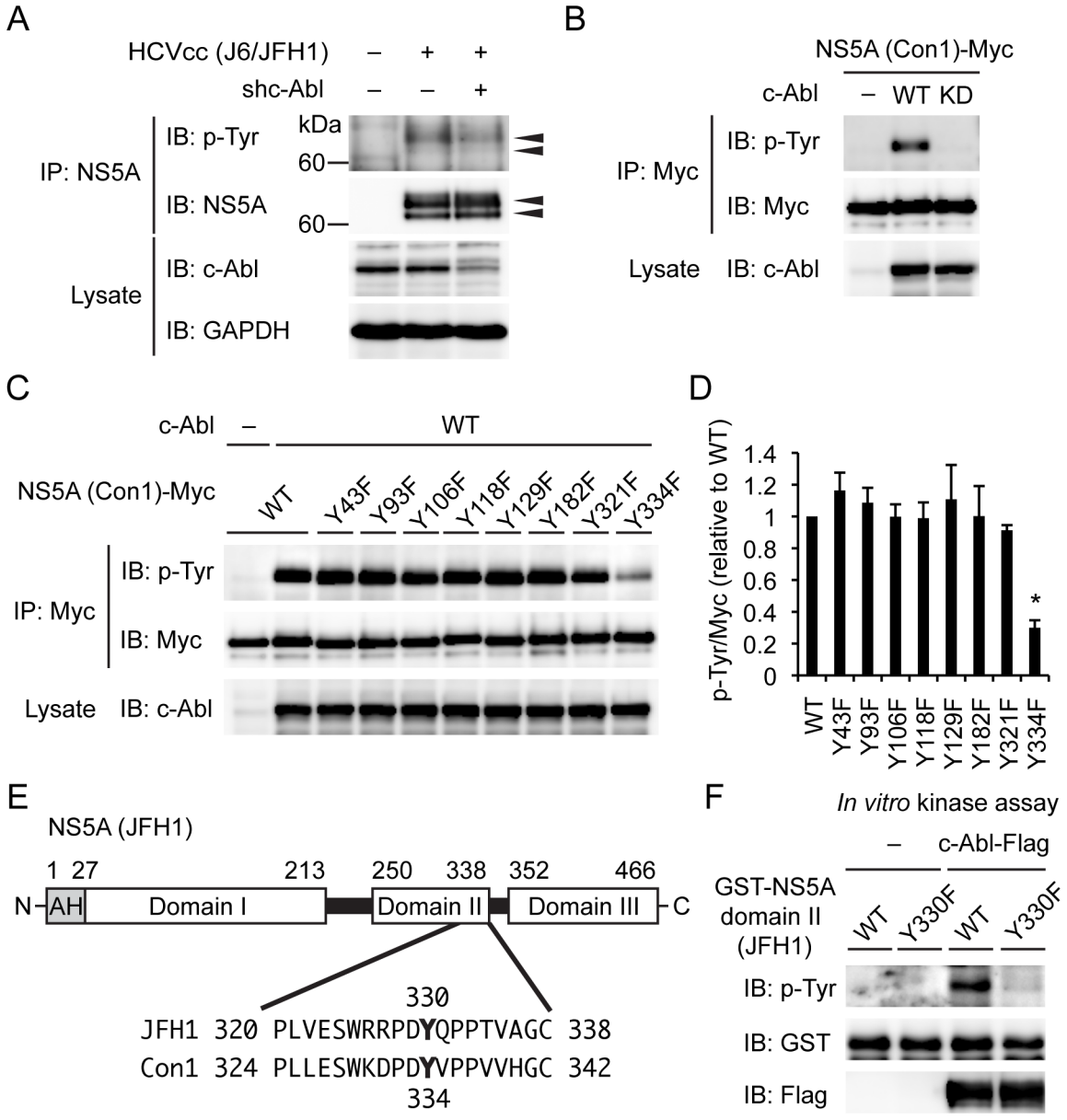

A
HCVcc (J6/JFH1) – + +
shc-Abl – – +
kDa
IP: NS5A
IB: p-Tyr
60
IB: NS5A
60
Lysate
IB: c-Abl
IB: GAPDH
B
NS5A (Con1)-Myc
c-Abl – WT KD
IP: Myc
IB: p-Tyr
IB: Myc
Lysate
IB: c-Abl
C
c-Abl – WT
NS5A (Con1)-Myc
WT Y43F Y93F Y106F Y118F Y129F Y182F Y321F Y334F
IP: Myc
IB: p-Tyr
IB: Myc
Lysate
IB: c-Abl
D
p-Tyr/Myc (relative to WT)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT Y43F Y93F Y106F Y118F Y129F Y182F Y321F Y334F
E
NS5A (JFH1)
1 27 213 250 338 352 466
N AH Domain I Domain II Domain III C
330
JFH1 320 PLVESWRRPDYQPPTVAGC 338
Con1 324 PLLESWKDPDYVPPVVHGC 342
334
F
In vitro kinase assay
– c-Abl-Flag
GST-NS5A domain II (JFH1)
WT Y330F WT Y330F
IB: p-Tyr
IB: GST
IB: Flag

FIGURE 4

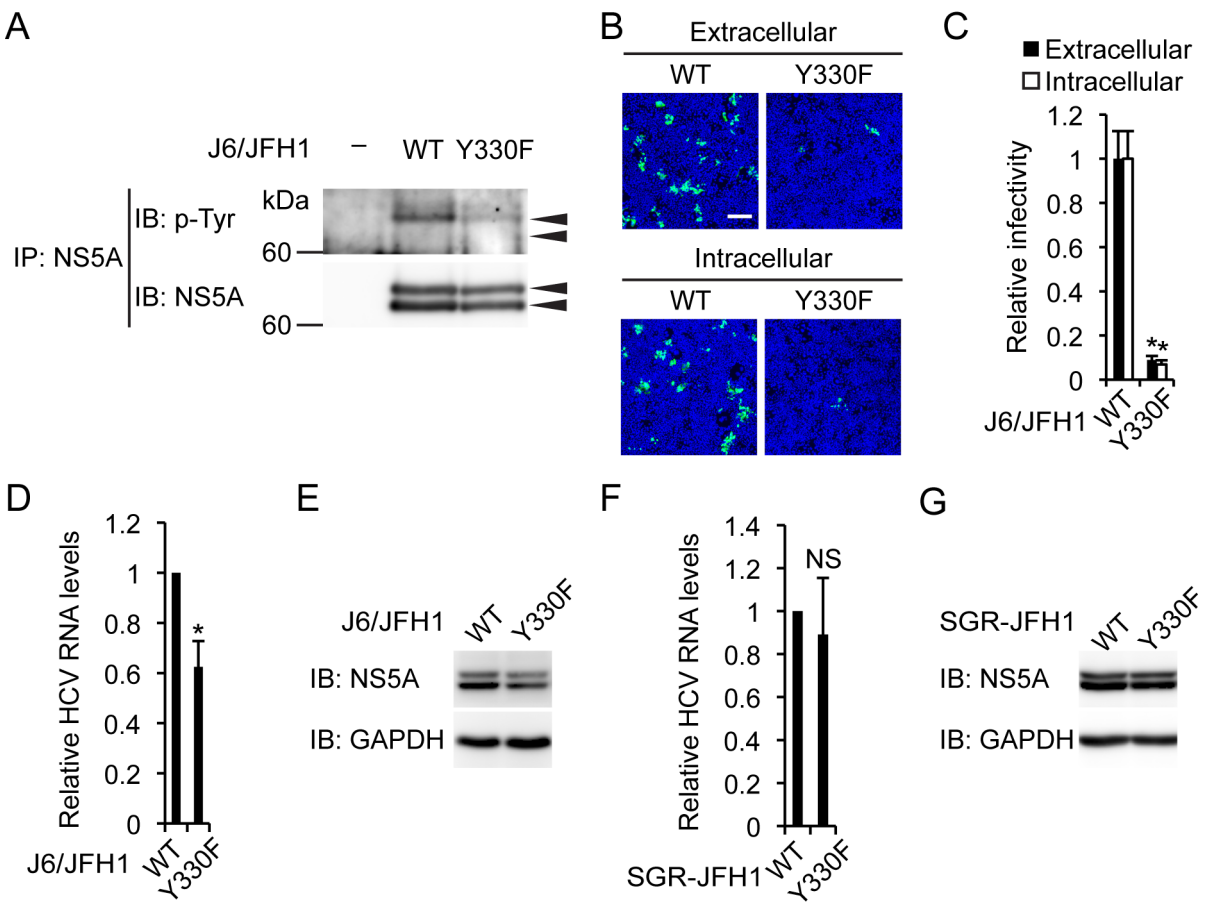

A
J6/JFH1
–
WT
Y330F
kDa
IP: NS5A
IB: p-Tyr
IB: NS5A
60
60
B
Extracellular
WT
Y330F
Intracellular
WT
Y330F
C
Extracellular
Intracellular
Relative infectivity
1.2
1
0.8
0.6
0.4
0.2
0
**
J6/JFH1
WT
Y330F
D
Relative HCV RNA levels
1.2
1
0.8
0.6
0.4
0.2
0
*
J6/JFH1
WT
Y330F
E
J6/JFH1
WT
Y330F
IB: NS5A
IB: GAPDH
F
Relative HCV RNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
SGR-JFH1
WT
Y330F
G
SGR-JFH1
WT
Y330F
IB: NS5A
IB: GAPDH

FIGURE 5

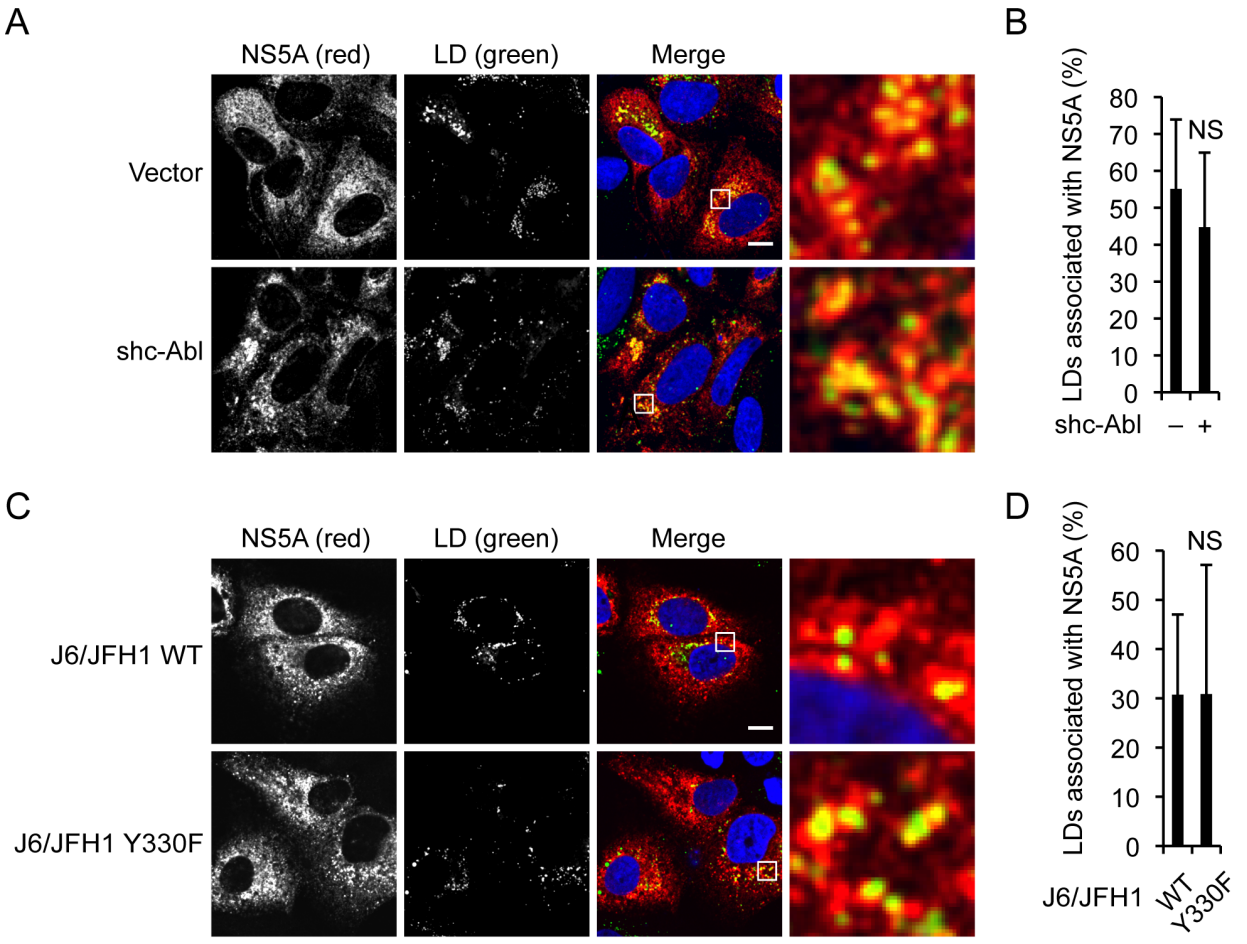
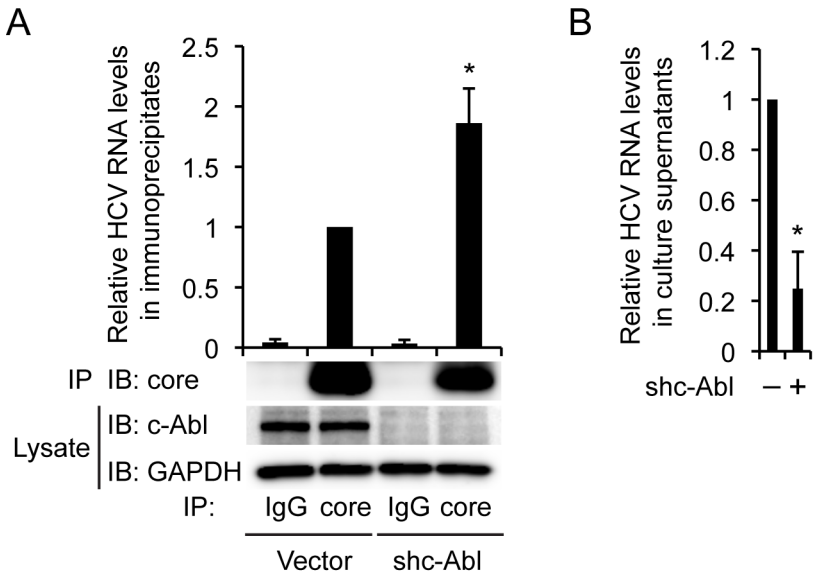

A
NS5A (red)
LD (green)
Merge
Vector
shc-Abl
B
LDs associated with NS5A (%)
80
70
60
50
40
30
20
10
0
NS
shc-Abl – +
C
NS5A (red)
LD (green)
Merge
J6/JFH1 WT
J6/JFH1 Y330F
D
LDs associated with NS5A (%)
60
50
40
30
20
10
0
NS
J6/JFH1 WT Y330F

FIGURE 6


A
Relative HCV RNA levels in immunoprecipitates
2.5
2
1.5
1
0.5
0
*
IP IB: core
Lysate
IB: c-Abl
IB: GAPDH
IP: IgG core IgG core
Vector
shc-Abl
B
Relative HCV RNA levels in culture supernatants
1.2
1
0.8
0.6
0.4
0.2
0
*
shc-Abl – +